AD_____

GRANT NUMBER DAMD17-94-J-4463

TITLE: The Role of Basic Fibroblast Growth Factor in Human Breast Cancer

PRINCIPAL INVESTIGATOR: Robert Wieder, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Medicine and Dentistry of New Jersey
Newark, New Jersey 07103

REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

WIEDER, ROBERT

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 Sep 96 - 31 Aug 97)

4. TITLE AND SUBTITLE
The Role of Basic Fibroblast Growth Factor in Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4463

6. AUTHOR(S)
Robert Wieder, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Medicine and Dentistry of New Jersey
Newark, New Jersey 07103

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

We have demonstrated that recombinant basic fibroblast growth factor (bFGF) inhibits proliferation and promotes programmed cell death in MCF-7 and a number of other breast cancer cells. These effects are opposite of the effects seen in fibroblasts. Since bFGF acts as a key angiogenesis factor in breast cancer and the bFGF content of breast cancer cells diminishes as they progress toward an ever more malignant phenotype, we investigated the effects of overexpressing bFGF in MCF-7 cells on the survival of these cells following treatment with two chemotherapeutic agents, etoposide and 5-fluorouracil. We contrasted these finding with data obtained in NIH 3T3 fibroblasts also transduced with bFGF. The data demonstrate that while the expression of even low levels of bFGF in NIH 3T3 fibroblasts protects from serum deprivation and etoposide or 5-fluorouracil-induced programmed cell death, the effect is opposite in MCF-7 cells where all moieties of bFGF promote apoptosis and enhance the effects of these chemotherapeutic agents. Our data demonstrate opposite regulation of Bcl-2 by overexpression of bFGF in these two cell types that correlate with the phenotypic findings.

14. SUBJECT TERMS
Breast Cancer
apoptosis, MCF-7 cells, NIH 3T3 cells, etoposide, 5-fluorouracil, Bcl-2, Bax

15. NUMBER OF PAGES
20

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

✓ Where copyrighted material is quoted, permission has been obtained to use such material.

✓ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

✓ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

___ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI Signature 9/3/97 Date

TABLE OF CONTENTS

THE ROLE OF BASIC FIBROBLAST GROWTH FACTOR IN HUMAN BREAST CANCER

Annual report, September 30, 1997

INTRODUCTION

We have previously demonstrated that basic fibroblast growth factor (bFGF, FGF-2), a classical mitogen and survival factor in fibroblasts and endothelial cells, inhibits proliferation in MCF-7 and several other breast cancer cell lines. In MCF-7 cells, bFGF binds high affinity FGF receptors, activates the MAP kinase pathway, induces both mitogenic and inhibitory events in the G_1 phase of the cell cycle and results in cell cycle arrest (1, 2).

We have also demonstrated that bFGF acts paradoxically to promote programmed cell death in MCF-7 cells. It induces a decrease in colony survival in tissue culture and an increase in apoptosis, as determined by morphologic criteria and flow cytometric quantitation of FITC-labeled dUTP 3'-OH DNA end labeling. The promotion of cell death was associated with a decrease in Bcl-2 mRNA and protein levels and an increase in Bax protein levels. Co-immunoprecipitation of Bcl-2 and Bax demonstrated a relative decrease in the fraction of total Bcl-2 in the homodimeric state and an increase in the fraction of Bcl-2 heterodimerized with Bax. Analogously, there was a decrease in the fraction of total Bax in the heterodimeric state with Bcl-2 and an increase in the fraction of Bax in its homodimeric form.

Since bFGF plays a role in mammary tumor formation and mammary epithelial cells lose intracellular bFGF by secretion and later stop synthesizing it, we sought to determine if overexpression of bFGF in MCF-7 cells also played a paradoxic role to that of bFGF in NIH 3T3 fibroblasts with respect to cell survival. The data presented in this report outline our contrasting observations in NIH 3T3 cells and MCF-7 breast cancer cells transduced with retroviral vectors that express bFGF. These observations may lend support to a role for bFGF in the progressive chemoresistance of breast cancer cells as they dedifferentiate and lose intracellular bFGF.

BODY

Effect of bFGF expression on survival of NIH 3T3 cells

We constructed NIH 3T3-derived cell lines expressing human bFGF using retroviral gene transfer with an N2-based vector (figure 1). One G418^R N2-transduced NIH 3T3 cell line and three G418^R NCF-transduced NIH 3T3 cell lines were chosen for study based on their bFGF levels. Southern blot analysis of restriction endonuclease XbaI-digested genomic DNA probed with a bFGF cDNA fragment showed that intact vector DNA of the expected size (4955 bp) was present in the NCF-transduced cells (figure 2).

Figure 1

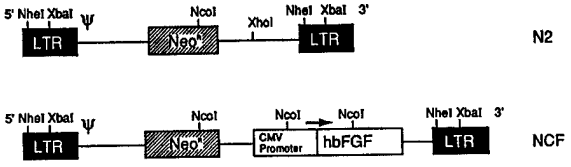


Figure 2

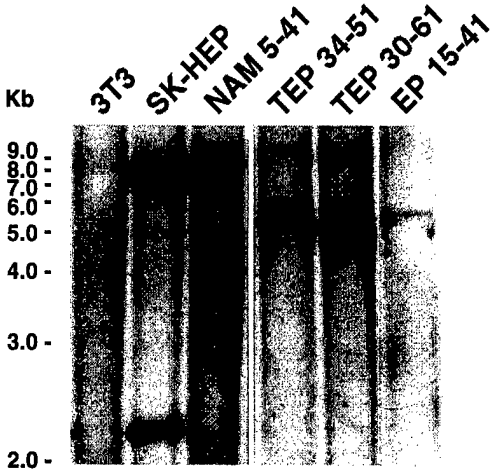


Figure 1. Retroviral vectors used to transduce NIH 3T3 cells. N2 is a Moloney Leukemia Virus-based vector in which the viral genes are replaced by a bacterial neomycin phosphotransferase gene (Neo^r) (3). NCF contains an immediate-early CMV promoter and a human bFGF cDNA cloned into the Xho I site of N2.

Figure 2. Southern blot analysis of restriction endonuclease Xba I-cleaved genomic DNA from uninfected (3T3), N2-infected (NAM 5-41), and NCF-infected (TEP 34-51, TEP 30-61 and EP 15-41) cell lines probed with a ^{32}P-labeled 432 bp fragment of bFGF cDNA. The hepatoma cell line SKHep-1, known to overproduce bFGF was used as a positive control. A 1 kb ladder (Gibco, BRL) was used as a size marker.

The cell lines selected for studies, TEP 34-51, TEP 30-61 and EP 15-41 contained from 2 to 14 times the amounts of immunoreactive bFGF on ELISA than NAM 5-41, the N2-transduced 3T3 cells (Table 1). The bFGF in the transduced cell lines was demonstrated to be biologically active by the plasminogen activator assay (not shown). Western blot determinations of bFGF using Protein A sepharose purified anti-rhbFGF IgG revealed at least three species of immunoreactive bFGF on SDS PAGE gels (figure 3). The three species were the 18 kDa moiety which is the AUG-initiated protein, and the 22 and 24 kDa (4, 5) proteins which are the CUG initiated moieties. The relative abundance of bFGF bands on Western blot were similar in the various cell lines. TEP 30-61 and EP 15-41 had the most abundant species, while TEP 34-51 had bands which were distinguishable at the limit of detection (1 ng 18 kDa bFGF) in the Western blot. SK-HEP 1 cells were used as a positive control. NIH 3T3, NAM 5-41 and EP 16-11 (an NCF-transduced NIH 3T3 control cell line which did not produce higher than background levels of bFGF) cells had undetectable bands of these molecular weights. The three bFGF-

specific bands of approximate M_r 18, 22 and 24 in SK-HEP 1, TEP 34-51, EP 15-41 and TEP 30-61 cells disappeared when blots were reprobed with antibody incubated with exogenous bFGF 10 μg/ml (data not shown). Nonspecific bands which appear at approximate M_r 17.5, 22.5 and 23 and higher (not shown) remained visible when the blot was restained with anti-human bFGF antibody that had been blocked by exogenous bFGF. The nonspecific bands varied in intensity from gel to gel, and depended on the stringency of washing. The values of bFGF obtained by ELISA for NIH 3T3 cells and NAM 5-41 cells are below the level of detection by Western. The Western data correlated with the bFGF values measured by ELISA for the transduced cell lines. In turn, these values correlated qualitatively with the relative bFGF mRNA levels detected in the different transduced cell lines.

TABLE 1

BASIC FGF CONTENT OF THE CELL LINES MEASURED BY ELISA

Cell line	ng bFGF/10^6 cell
NIH 3T3	0.151 $\pm$ 0.013
NAM 5-41	0.211 $\pm$ 0.029
TEP 34-51	0.407 $\pm$ 0.010
EP 15-41	3.028 $\pm$ 0.087
TEP 30-61	1.725 $\pm$ 0.094

Figure 3

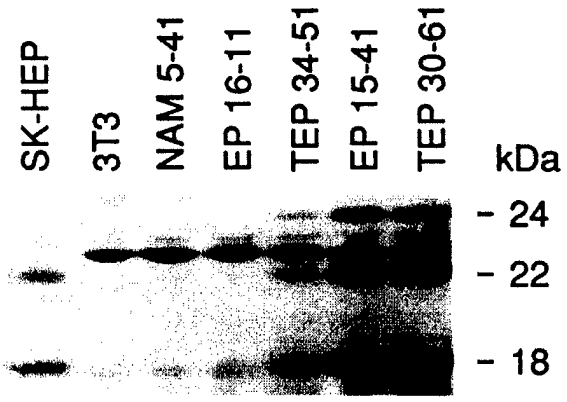


Figure 3. Western blot of cellular bFGF from SKHep-1, untransduced (3T3), N2-transduced and NCF-transduced (EP 16-11, TEP 34-51, EP 15-41 and TEP 30-61) cells. EP 16-11 cells did not contain bFGF measurable above background in an ELISA and are included as a control. One hundred μg of protein from each cell line was electrophoresed in a 12.5% SDS PAGE system, electroblotted to Immobilon PVDF transfer membranes (Millipore, Bedford MA) and probed with Protein A sepharose-purified anti hbFGF rabbit IgG. Exogenous rhbFGF co-migrated with the 18 kDa band and was detectable at 1 ng. bFGF-specific bands disappeared with co-incubation of the antibody with 10 μg/ml bFGF.

These data demonstrate that NIH 3T3-derived cell lines were constructed which contained single copies of the bFGF vectors and expressed bFGF mRNA and bioactive 18, 22 and 24 kD bFGF protein of concentrations 2 to 15-fold higher than in an N2-transduced control cell line. Using these cell lines, we proceeded to investigate phenotypic effects of bFGF overexpression.

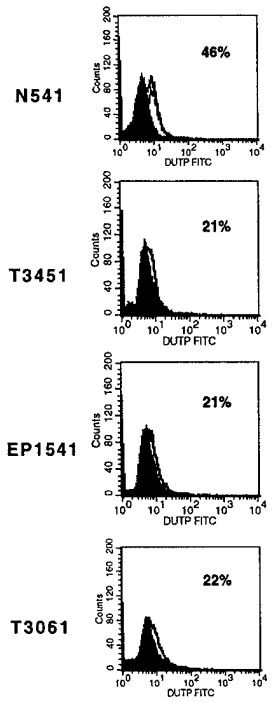
Modulation of apoptosis and survival in NIH 3T3 cells

In order to determine if the overexpression of bFGF contributed to the transformed phenotype by altering the capacity of cells to undergo programmed cell death, we determined serum deprivation-induced apoptosis in the transduced NIH 3T3 cell lines. Fluorescence sorting of cells containing dUTP-FITC 3'-OH end-labeled DNA was carried out 24 hours after serum deprivation. Figure 4 demonstrates that cell lines TEP 34-51, EP 15-41 and TEP 30-61 had lower increases in fluorescence intensity reflecting DNA end-labeling after 24 hours of serum deprivation than control cell line NAM 5-41 when compared to the cells incubated in standard

media containing 10% fetal calf serum. After 24 hours of serum deprivation, TEP 34-51, EP 15-41 and TEP 30-61 cells had increases in fluorescence intensity of 21%, 21% and 22% as compared with 46% for NAM 5-41 cells. These data translate to a delay of apoptosis, with higher quantities of DNA fragmentation observed with longer serum-free incubations. Antibody to bFGF did not reverse the protective effects of endogenous overexpression of bFGF (data not shown). The basal levels of DNA fragmentation measured by 3'-OH FITC-labeled dUTP in rapidly proliferating cells in the presence of 10% fetal calf serum did not differ among the cell lines expressing variable amounts of bFGF.

Genomic DNA prepared from NIH 3T3 and NAM 5-41 cells grown in serum-free DME for 24 hours (6) showed a pattern of a 200 base pair ladder consistent with apoptosis (figure 5). TEP 34-51 and TEP 30-61 cells contained exclusively high molecular weight genomic DNA. NIH 3T3 and NAM 5-41 cells prepared by cytospin following these incubations showed marked alterations in morphology, including cytoplasmic bleb formation and nuclear fragmentation as compared to TEP 34-51 and TEP 30-61 cells which were predominantly intact (data not shown).

Figure 4 **Figure 5**



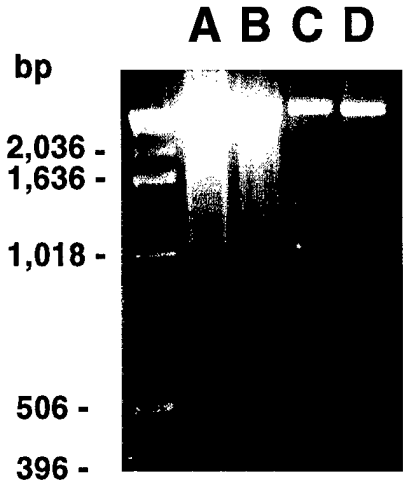


Figure 4. Fluorescence sorting of cells containing dUTP-FITC 3'-OH end-labeled DNA was carried out 24 hours after serum deprivation. The data demonstrate that cell lines TEP 34-51, EP 15-41 and TEP 30-61 had lower increases in fluorescence intensity reflecting DNA end-labeling after 24 hours of serum deprivation than control cell line NAM 5-41 when compared to these cells incubated in standard media containing 10% fetal calf serum. Numbers shown represent percent shifts in the geometric mean of the fluorescence intensity of the area under the curves between serum-deprived cells and the same cells grown in 10% FCS.

Figure 5. Apoptosis of selected cell lines, as measured by agarose gel electrophoresis of DNA isolated from uninfected, N2-transduced and two NCF-transduced NIH 3T3 cell lines grown under conditions of serum starvation. Confluent cells were switched to serum-free DMEM, 0.5% endotoxin-free BSA, 2 mM glutamine for 24 hours. Nonadherent cells were collected and DNA was prepared. 3 μg of genomic DNA was electrophoresed in 1.15% agarose in 1 μg/ml ethidium bromide. Left hand lane: 1 kb marker, lane A: NIH 3T3, lane B: NAM 5-41, lane C: TEP 34-51 and lane D: TEP 30-61 cells.

To determine if the effect of preventing programmed cell death translates to cellular survival, a clonogenic assay was carried out to determine the protective effects of bFGF from two chemotherapeutic agents with different mechanisms of action. Figure 6 demonstrates that the decreased efficiency of colony formation after one hour treatment with variable doses of etoposide (A), a topoisomerase II inhibitor or 5-fluorouracil (B), an antimetabolite, observed in NIH 3T3 cells and NAM 5-41 cells was prevented in NIH 3T3-derived cells overexpressing bFGF. The lower panels expressing the data as a percentage decrease in colony number compared to untreated cells underscores this effect.

Figure 6

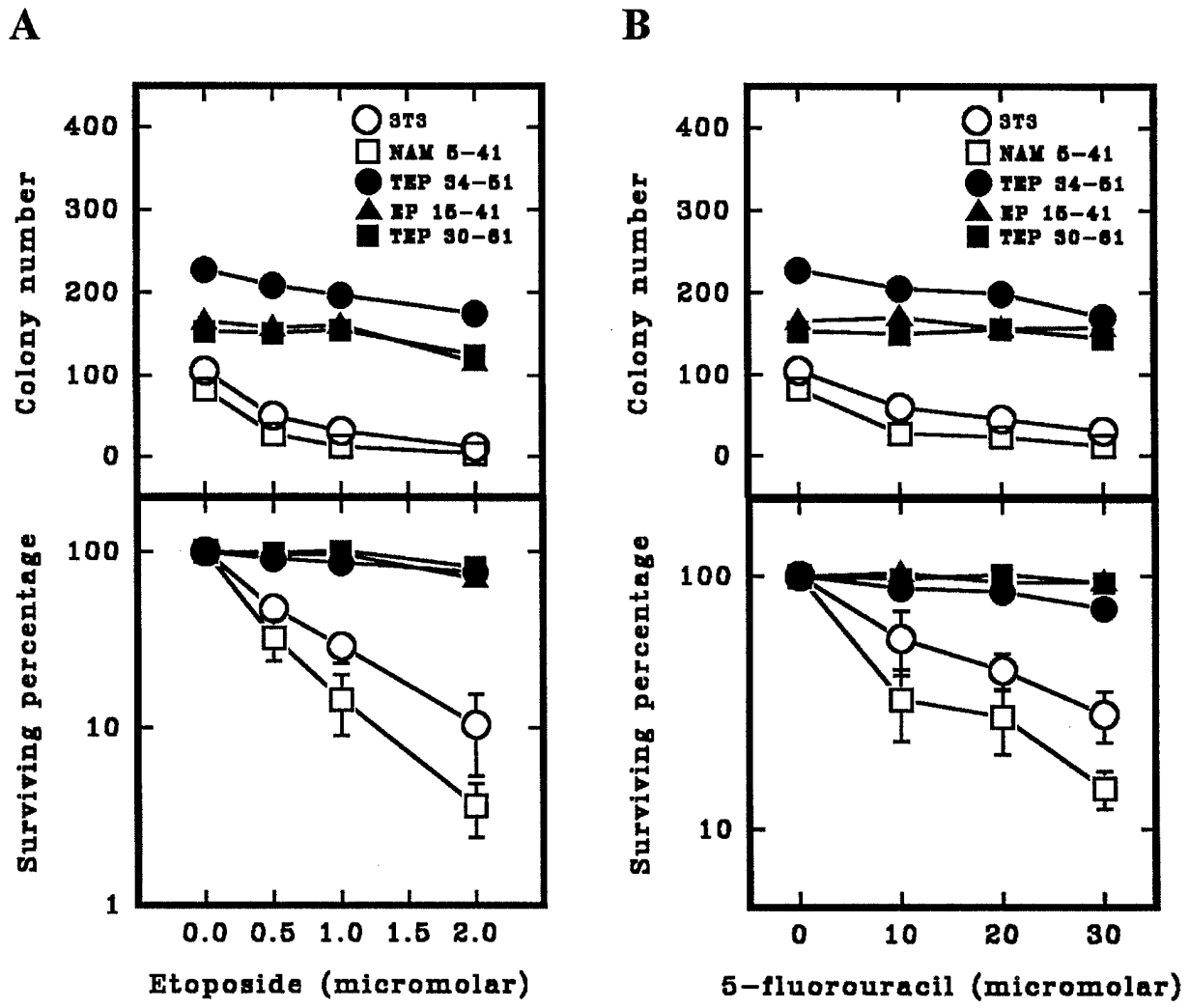


Figure 6. Effects of bFGF overexpression on the clonogenicity of NIH 3T3 cells treated with variable doses of etoposide (A) and 5-FU (B) for one hour in day 12±2 colonies. Cell lines overexpressing bFGF, TEP 34-51, EP 15-41 and TEP 30-61, had increased baseline colony formation and were resistant to lowering of clonogenic potential by the drugs at the concentrations shown. Lower panels demonstrate percentage decreases in colony formation.

These data demonstrate that cells overexpressing a two-fold or higher increase of bFGF levels over background are more resistant to serum deprivation-induced apoptosis and to chemotherapy-induced loss of clonogenic potential than control cells, while overexpression of bFGF does not affect the baseline apoptotic rate of cells growing in serum-replete media. We proceeded to determine cellular levels of Bcl-2 and Bax proteins to obtain data supporting one possible mechanism for these observations.

Effects of bFGF expression in NIH 3T3 cells on cellular Bcl-2 and Bax content

Western immunoblots of the three cell lines overexpressing bFGF, the NAM 5-41 control cell line and the 3T3 cell population were carried out on 100 μg protein from lysates of cells grown to confluence in DMEM, 10% FCS and subsequently serum deprived for 24 hours. The electrophoresed proteins were transferred to PVDF membranes and stained with antibody to Bcl-2 and Bax. The blots demonstrated that all three cell lines overproducing bFGF, TEP 34-51, EP 15-41 and TEP 30-61, contained higher levels of intracellular Bcl-2 than the N2-transduced control cell line and the 3T3 cell population (figure 7A). Immunostaining with anti-Bax antibody, however, demonstrated no differences in Bax content among the cells. These data demonstrate that Bcl-2 protein levels are elevated in NIH 3T3 cells overexpressing as little as two-fold higher bFGF levels than controls, while cellular Bax levels are not altered by bFGF overexpression. The expression of higher cellular levels of Bcl-2 correlated with the relative resistance to apoptosis in these cells. Antibody to bFGF did not reverse the overexpression of Bcl-2 in cells overexpressing bFGF (figure 7B), corresponding to the lack of an observed reversal of their relative resistance to serum deprivation-induced apoptosis described earlier.

Figure 7

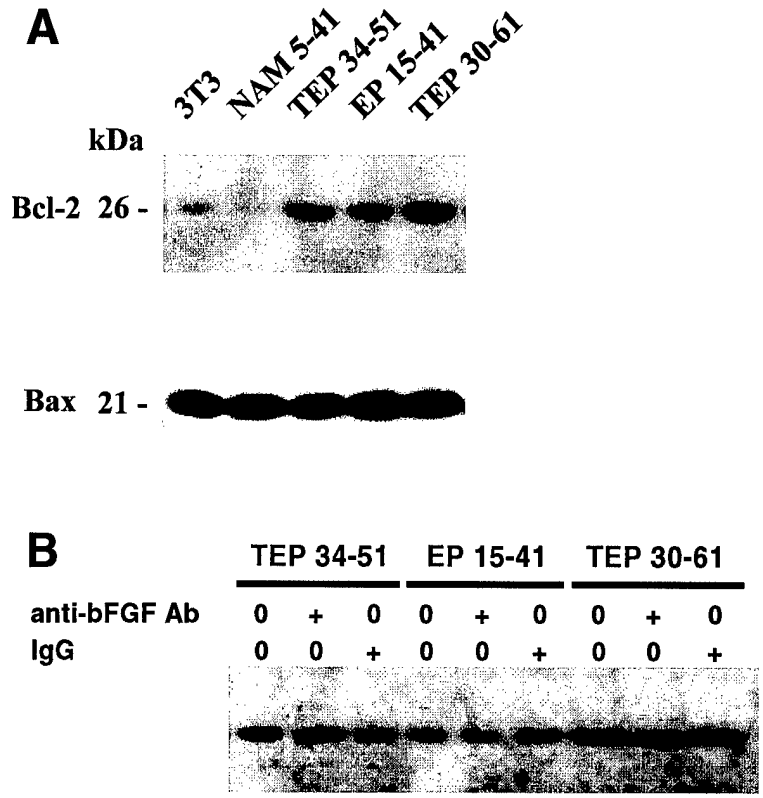


Figure 7. A. Western blot of cellular Bcl-2 and Bax. One hundred μg of protein from each cell line were electrophoresed in 12.5% SDS PAGE systems, electroblotted to Immobilon PVDF transfer membranes (Millipore, Bedford MA). Membranes were probed with either antibody to rodent Bcl-2 (PharMingen, San Diego, CA) or Bax (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Bound antibody was detected using the ECL chemiluminescence detection system (Amersham, Arlington Heights, IL). B. Western blot of bFGF-producing cell lines incubated with antibody to bFGF or IgG.

Effect of bFGF expression on survival of MCF-7 cells

MCF-7 cells were transduced with retroviral vectors encoding the 18, 22 and 24 kD bFGF species (NCF) that localized both in the cytoplasm and the nucleus, only the 18 kD species (ΔA) or with the parental vector N2 (figure 8) as previously described in a prior annual report. Southern blots of genomic DNA from transduced cell populations selected with G418 digested with endonuclease Xba I were hybridized with a ^{32}P-labeled *bFGF* cDNA fragment. The blot showed single bands corresponding to the intact NCF vector of 4955 base pairs and the ΔA vector of 4648 base pairs, spanning distances between the two LTR Xba I sites (figure 9). When probed with a fragment of the *Neo* gene, intact N2 vector of 3257 base pairs was also seen in the N2-transduced cells (not shown). High molecular weight signals representing genomic bFGF sequences were present in all three cell types when probed with the *bFGF* probe.

Figure 8

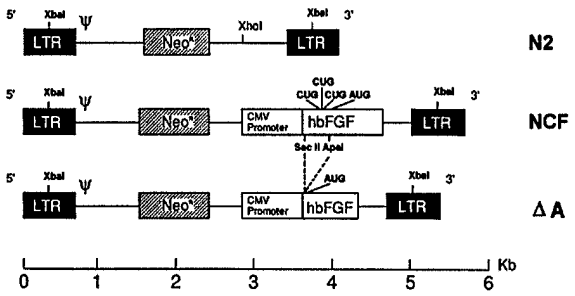


Figure 9

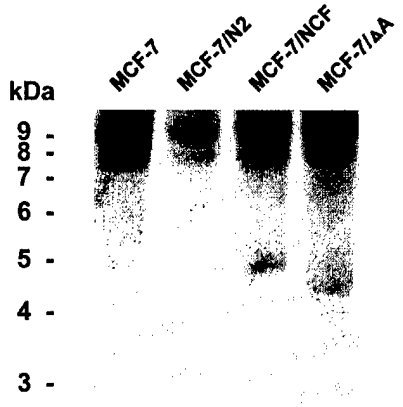


Figure 8. Retroviral vectors used to transduce MCF-7 cells. NCF contains an immediate-early CMV promoted human *bFGF* cDNA cloned into the Xho I site of N2. A Sac II/Apa I fragment was deleted from NCF to construct ΔA.

Figure 9. Southern blot analysis of restriction endonuclease Xba I-cleaved genomic DNA from MCF-7 cells transduced with N2, NCF and ΔA. Ten μg restriction endonuclease-digested DNA samples were electrophoresed in 1% agarose, transferred to nylon membranes and probed with a ^{32}P-labeled 432 bp endonuclease Bam H1 fragment of *bFGF* cDNA spanning positions -27 to +406 of the cDNA coding sequence.

Effects of bFGF expression on colony-forming potential

To demonstrate that overexpression of bFGF decreases cell survival, we carried out clonogenic assays in tissue culture, as before (7). Two thousand MCF-7/N2, MCF-7/NCF or MCF-7/ΔA cells were incubated in 60 mm dishes in standard media for 24 hours, then for one hour with 0, 0.25, 0.5 or 1.0 μM etoposide (figure 10A) or 0, 50, 100 or 200 μM 5-fluorouracil (figure 10C) or for 0, 0.5 1.0 or 2.0 hours with 1.0 μM etoposide (figure 10B) or 100 μM 5-fluorouracil (figure 10D). Colonies were stained with methylene blue and counted manually after 12 ± 2 days. One hour incubations with etoposide from 0.25 to 1 μM resulted in a maximum reduction of 40% in clonogenic potential in MCF-7/N2 cells (figure 10A). Similarly, one hour incubations with 5-fluorouracil concentrations from 50 to 200 μM caused a maximum reduction of 53% in colonies in these control cells (figure 10C). MCF-7/NCF cells expressing all forms of bFGF had 28-43% less colonies and MCF-7/ΔA cells expressing the 18 kD bFGF moiety had 33-37% less colonies at baseline than N2 transduced control MCF-7 cells. One hour incubations with etoposide concentrations from 0.25 to 1.0 μM caused a further

maximum reduction of colony formation of 32% in MCF-7/NCF cells and 29% in MCF-7/ΔA cells (figure 10A) while 0.5 to 2 hour incubations with 1.0 μM etoposide caused a further maximum reduction of colony formation of 35% in MCF-7/NCF cells and 48% MCF-7/ΔA cells (figure 10B). Similarly, one hour incubations of with 5-fluorouracil concentrations from 50 to 200 μM caused a further maximum reduction in colony formation of 44% in both MCF-7/NCF and MCF-7/ΔA cells (figure 10C) while 0.5 to 2 hour incubations with 100 μM 5-fluorouracil caused a further maximum reduction in colony formation of 48% in MCF-7/NCF cells and 46% MCF-7/ΔA cells (figure 10D). All of the differences cited above were significant at $p < 0.001$. The overlapping curves in the lower panels of figure 1 denoting decreases in the percentage of colonies formed after treatment of different cell types with variable doses and times of chemotherapeutic drugs suggest that the effects of bFGF overexpression were additive with those of the chemotherapeutic agents (7).

Figure 10

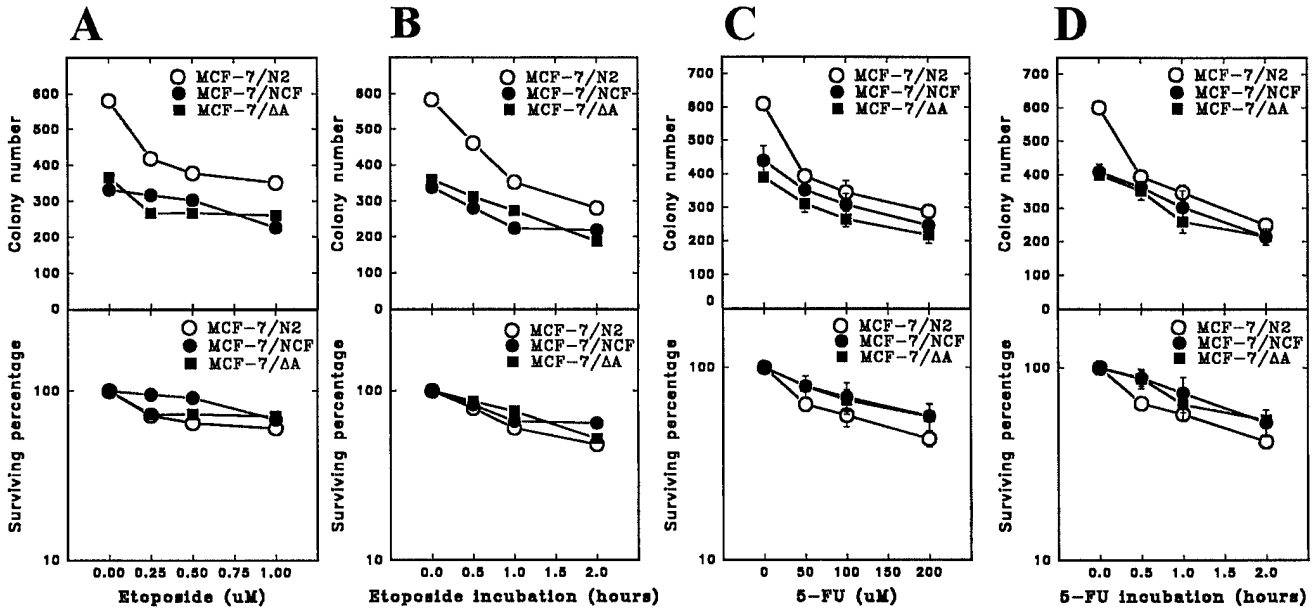


Figure 10. Effects of overexpression of 18 kD bFGF species (ΔA) or the 18 as well as the 22 and 24 kd species (NCF) as compared to infection with the N2 control vector on the clonogenicity of MCF-7 cells treated with variable doses of etoposide (A) and 5-FU (C) for one hour or for variable times with 1 μM etoposide (B) and 100 μM 5-FU (D). Lower panels demonstrate percentage decreases in colony formation. Overlapping curves denote additive effects.

Effects of bFGF expression on apoptosis

The above data demonstrated that overexpression of all forms of bFGF in MCF-7 cells induces inhibition of colony forming ability. This effect may be due to the influence of several factors, including inhibition of proliferation, a characteristic we already demonstrated (Wieder), survival, or induction of cell death. To determine the effect of bFGF overexpression on programmed cell death or apoptosis we determined the effects of bFGF overexpression on the basal rate of apoptosis and on the susceptibility to killing by two chemotherapeutic agents with different mechanisms of action, etoposide, a topoisomerase inhibitor, and 5-fluorouracil, an anitmetabolite, using three different assays.

Figure 11 demonstrates the effects of bFGF on cell death as determined by morphologic criteria identified with apoptosis. Cells were cultured on microscope slides, exposed to etoposide

Figure 11

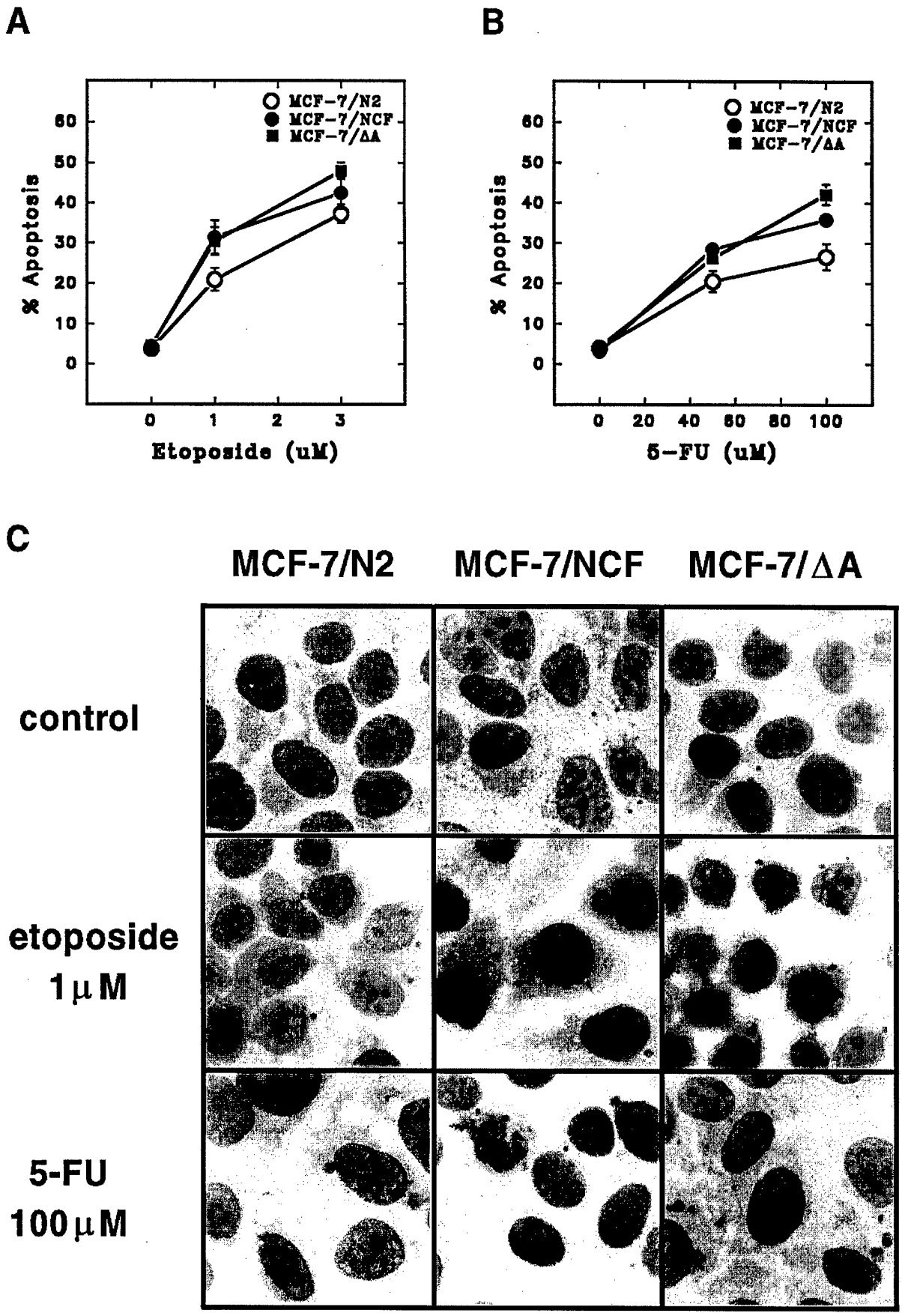

A
MCF-7/N2
MCF-7/NCF
MCF-7/ΔA
% Apoptosis
Etoposide (uM)
B
MCF-7/N2
MCF-7/NCF
MCF-7/ΔA
% Apoptosis
5-FU (uM)
C
MCF-7/N2
MCF-7/NCF
MCF-7/ΔA
control
etoposide 1μM
5-FU 100μM

Figure 11. A. Effects of bFGF expression on the rates of apoptosis induced by etoposide concentrations from 0 to 3 μM (A) or 5-fluorouracil 0-100 μM (B). (C) Morphologic appearance of MCF-7-derived cells, cells treated with etoposide, or cells treated with 5-FU photographed at 400X magnification demonstrating the greater pro-apoptotic effects of the chemotherapeutic agents in cells expressing bFGF.

0-3 μM or 5-fluorouracil 0-100 μM for one hour, Wright-Giemsa stained three days later and analyzed for morphologic criteria of apoptosis, including nuclear fragmentation, cytoplasmic vacuolation and nuclear and cell membrane blebbing using blinded 400 cell manual counts. Treatment of MCF-7/N2 cells with etoposide 1 and 3 μM increased the percentage of apoptotic cells from a baseline of 3.85 $\pm$ 0.86% to 20.92 $\pm$ 2.82% and 37.30 $\pm$ 2.30% ($p < 0.001$ for each) (figure 11A). While the basal apoptotic rate for the cells expressing bFGF was not significantly different from the control cells, treatment with etoposide 1 μM increased the percentage of MCF-7/NCF cells exhibiting apoptotic features to 31.43 $\pm$ 4.22% ($p < 0.01$) and the MCF-7/ΔA cells to 30.40 $\pm$ 3.46% ($p < 0.01$) as compared with the MCF-7/N2 cells. MCF-7/ΔA cells expressing the 18 kD bFGF moiety had a significant increase in cell death to 47.97 $\pm$ 2.07% as compared to MCF-7/N2 cells when treated with 3 μM etoposide. The increase in cell death of MCF-7/NCF cells treated with 3 μM etoposide to 42.4 $\pm$ 3.98% was not statistically significant ($p > 0.05$).

Treatment with 5-fluorouracil yielded significantly greater cell kill in both cell types expressing bFGF moieties than in the MCF-7/N2 controls at all of the concentrations tested (figure 11B). Treatment of MCF-7/N2 cells with 5-fluorouracil 50 μM and 100 μM yielded increases in the percentage of cell death from 4.03 $\pm$ 1.18% to 20.5 $\pm$ 2.62% ($p < 0.001$) and 26.7 $\pm$ 3.23% ($p < 0.001$), respectively. Treatment with 5-fluorouracil 50 μM increased the percentage of apoptotic MCF-7/NCF cells 28.54 $\pm$ 1.43% ($p < 0.01$) and the percentage of apoptotic MCF-7/ΔA cells to 26.30 $\pm$ 1.83% ($p < 0.02$) as compared with the MCF-7/N2 cells. MCF-7/NCF cells treated with 100 μM etoposide had an increased apoptotic rate of 35.93 $\pm$ 1.61% ($p < 0.01$ compared with MCF-7/N2 cells) and MCF-7/ΔA cells had an increase in cell death to 42.2 $\pm$ 2.57% ($p < 0.01$ as compared to MCF-7/N2). Figure 11C demonstrates the visible characteristics of the different cells treated with the two chemotherapeutic agents at selected concentrations of 1 μM etoposide and 100 μM 5-fluorouracil where the differences due to bFGF expression were most obvious.

TUNEL Assay

To confirm the findings that demonstrated a greater susceptibility to cell death in cells overexpressing bFGF by morphologic criteria, we repeated the experiments above using a TUNEL assay to determine rates of cell death. 5 x 10^5 cells were incubated in 60 mm dishes containing sterile microscope slide cover slips in standard media for 24 hours, the media was changed to standard media containing the chemotherapeutic drugs for one hour then back to standard media for three days at 37°C. The cells were permeabilized and the DNA was 3'-OH end labeled with FITC-dUTP using a Boehringer Manheim (Indianapolis, IN) Fluorescein In Situ Cell Death Detection Kit. Cells were photographed at 400x magnification with the Olympus BX40 fluorescence microscope and the PM20 photographic system. Figure 12 demonstrates that both cell types overexpressing bFGF had slightly greater background levels of DNA fragmentation than the control cells and that they both yielded to far greater drug induced DNA fragmentation than the control cells with both etoposide and 5-fluorouracil incubations.

Figure 12

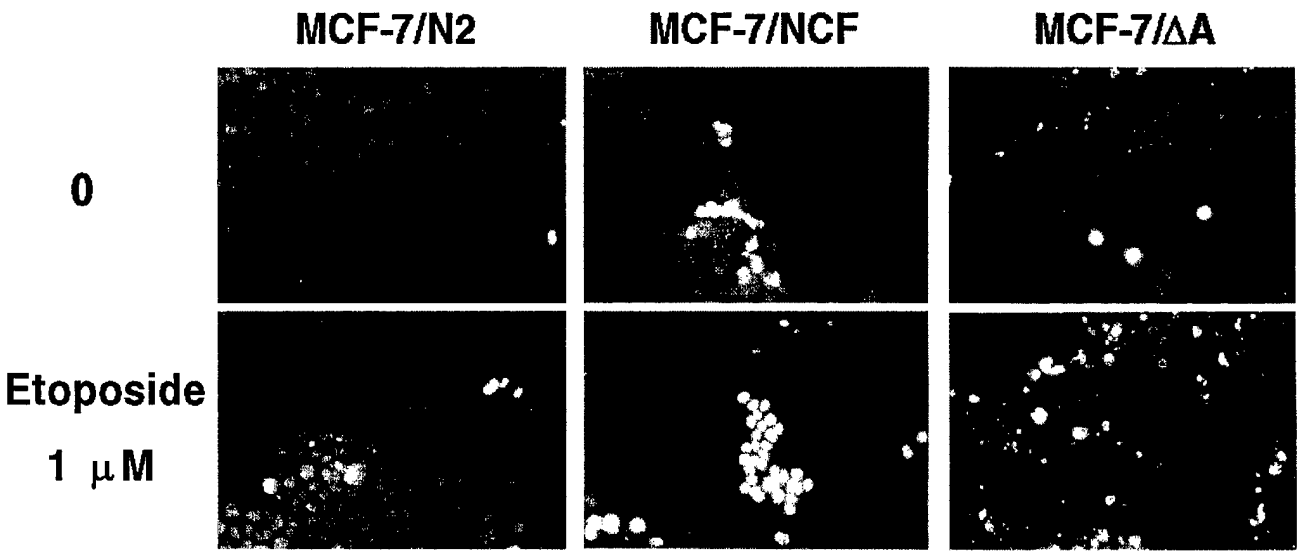


Figure 12. TUNEL assay of MCF-7-derived cells cultured on cover slips three days after a one hour incubation with and without 1 μM etoposide demonstrating increased susceptibility to apoptosis in cells overexpressing bFGF.

To quantitate these results, cells that were 3'-OH DNA end labeled with dUTP-FITC were analyzed by flow cytometry. The percentage shift in the geometric mean of the distribution of fluorescence labeling in all three cells types with and without treatment with etoposide or 5-fluorouracil was compared to untreated MCF-7/N2 cells that were used as the control reference point. Figure 13A demonstrates that treatment of MCF-7/N2 cells with etoposide from 0.5 to 3 μM induced a shift in the geometric mean of fluorescein labeling in the treated cells from 9.66% to 21.5%. The baseline incorporation of FITC in MCF-7/NCF and MCF-7/ΔA cells was shifted 19.87% and 39.08% respectively compared with MCF-7/N2 cells. Treatment with etoposide yielded greater shifts in the geometric mean of FITC-labeled MCF-7/NCF and MCF-7/ΔA cells at each concentration of etoposide than observed with MCF-7/N2 cells. The differences in the shifts in FITC labeling in the three cell types are depicted in figure 13B. Similarly, figure 13C demonstrates the increases in the geometric means of FITC labeling with increasing doses of 5-fluorouracil treatment in MCF-7/N2 cells, increases due to bFGF expression at baseline and greater susceptibility to FITC DNA end labeling at any given 5-fluorouracil concentration in either bFGF-expressing cell type as compared with MCF-7/N2 cells. Figure 13D depicts these differences in a graphic form. The extent of differences in the baseline shifts of FITC end labeling among the different cell types varied in different experiments. The trends, however, are consistent within each experiment carried out on any one day.

These data demonstrate that overexpression of bFGF promotes programmed cell death in MCF-7 cells. In order to determine if the mechanism for this observation is analogous to that observed in MCF-7 cells treated by exogenous recombinant bFGF, we analyzed the expression of Bcl-2 and Bax in these cells.

Figure 13. Flow cytometric analysis of MCF-7-derived cells treated with increasing doses of etoposide (A and B) or 5-FU (C and D) for one hour and labeled with FITC-dUTP by 3'-OH DNA terminal transferase three days after drug treatment. Bottom graphs visually depict the percent shifts in the geometric means of the fluorescence curves as functions of bFGF pretreatment and drug dose.

Figure 13

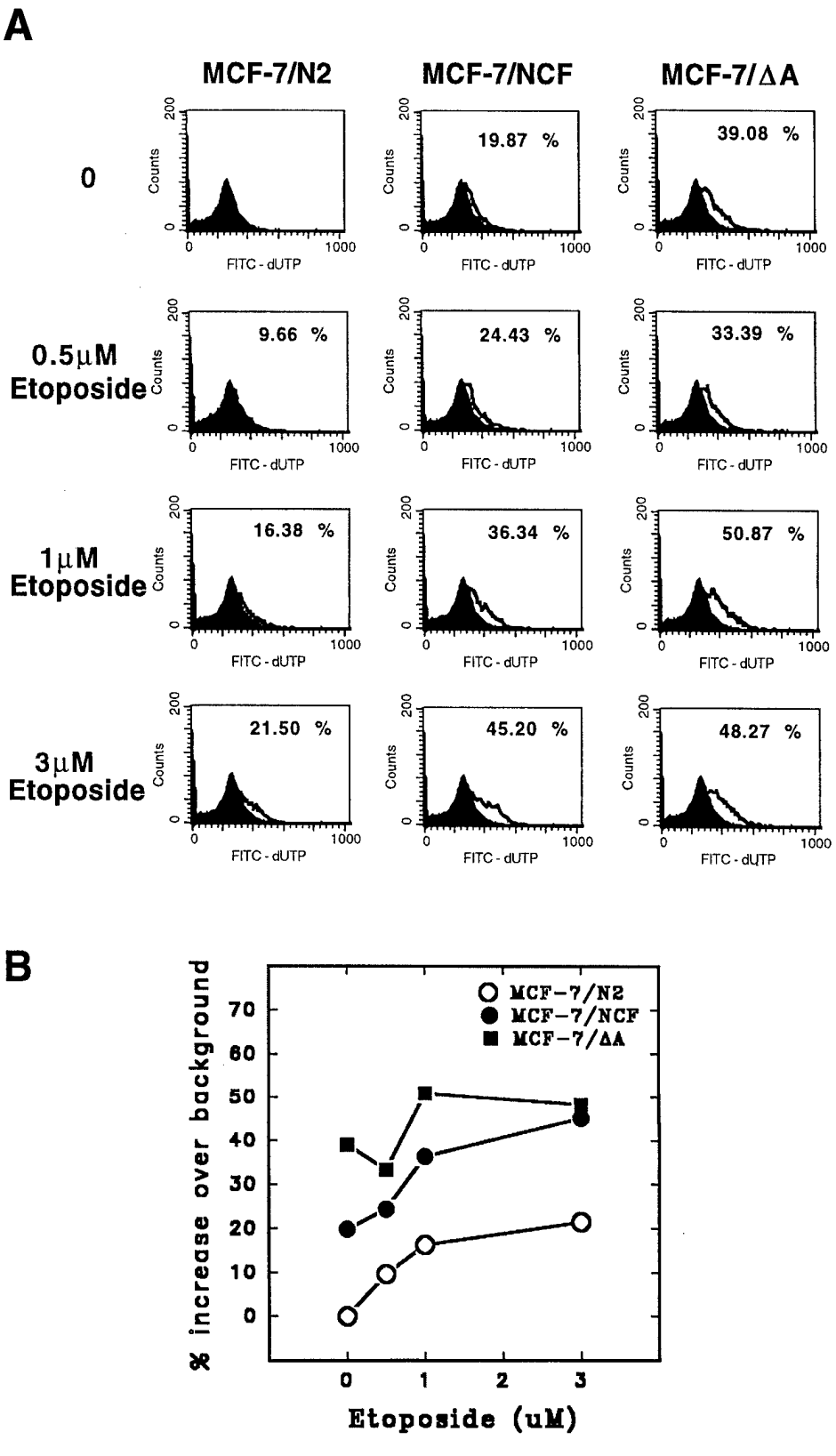
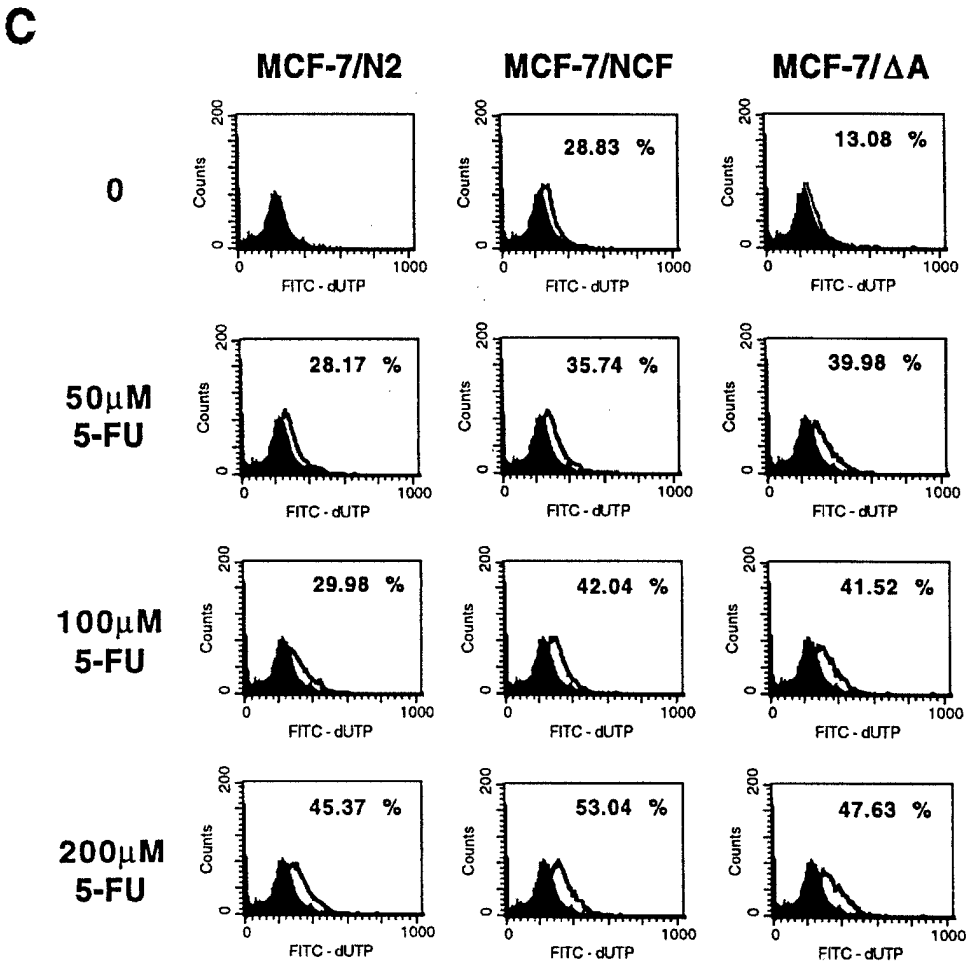

A
MCF-7/N2
MCF-7/NCF
MCF-7/ΔA
0
0.5μM Etoposide
1μM Etoposide
3μM Etoposide
Counts
FITC - dUTP
19.87 %
39.08 %
9.66 %
24.43 %
33.39 %
16.38 %
36.34 %
50.87 %
21.50 %
45.20 %
48.27 %
B
MCF-7/N2
MCF-7/NCF
MCF-7/ΔA
% increase over background
Etoposide (uM)

Figure 13


C
MCF-7/N2
MCF-7/NCF
MCF-7/ΔA
0
50μM 5-FU
100μM 5-FU
200μM 5-FU
28.83 %
13.08 %
28.17 %
35.74 %
39.98 %
29.98 %
42.04 %
41.52 %
45.37 %
53.04 %
47.63 %
Counts
FITC - dUTP
D
MCF-7/N2
MCF-7/NCF
MCF-7/ΔA
% increase over background
5-fluorouracil (uM)

Effects of bFGF expression on Bcl-2

Figure 14A demonstrates that Bcl-2 protein levels are decreased in MCF-7/NCF and MCF-7/ΔA cells while there don't appear to be any significant differences among the Bax levels in the two bFGF expressing cells and the control cells. The decrease in Bcl-2 levels in the bFGF expressing MCF-7 cells is also reflected at the mRNA level (figure 14B), suggesting that the decreased Bcl-2 protein is due to a decreased synthesis. It is not known at this time whether the decreased mRNA levels are due to decreased synthesis or stability. To determine a physiological significance for the decreased Bcl-2 protein levels, the relative ratio of Bcl-2 complexed with Bax was determined by co-immunoprecipitation. Cells were scraped from plates in 500 μl NP-40 lysis buffer, containing 50 mM Tris HCl pH 7,4, 150 mM NaCl, 0.5% NP-40, 1 mM $NaVO_4$, 1 mM dithiothreitol, 1 mM PMSF, 1 μg/ml leupeptin (Sigma, St. Louis, MO) and 0.01 U/ml aprotinin (8) and lysed by a 5 second sonication using a 4710 series Ultrasonics Homogenizer with a microtip (Cole Parmer, Instruments, Chicago, IL) at 4°C. 500 μg protein were incubated in 0.5 ml NP-40 buffer with 1 μg rabbit anti-Bax antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. The antibody-protein complexes were immunoprecipitated with 15 μl Protein G Plus/Protein A-Agarose (Oncogene Science, Uniondale, NY) and after a 2 hour 4°C incubation were centrifuged for 15 minutes at 2000 rpm, washed three times with NP-40 buffer, electrophoresed and analyzed by Western blot with monoclonal antibodies to Bcl-2 (Coulter Corp., Opa Locka, FL). Figure 14C demonstrates that there was significantly less Bcl-2 complexed with Bax in cells expressing both types of bFGF moieties.

Figure 14

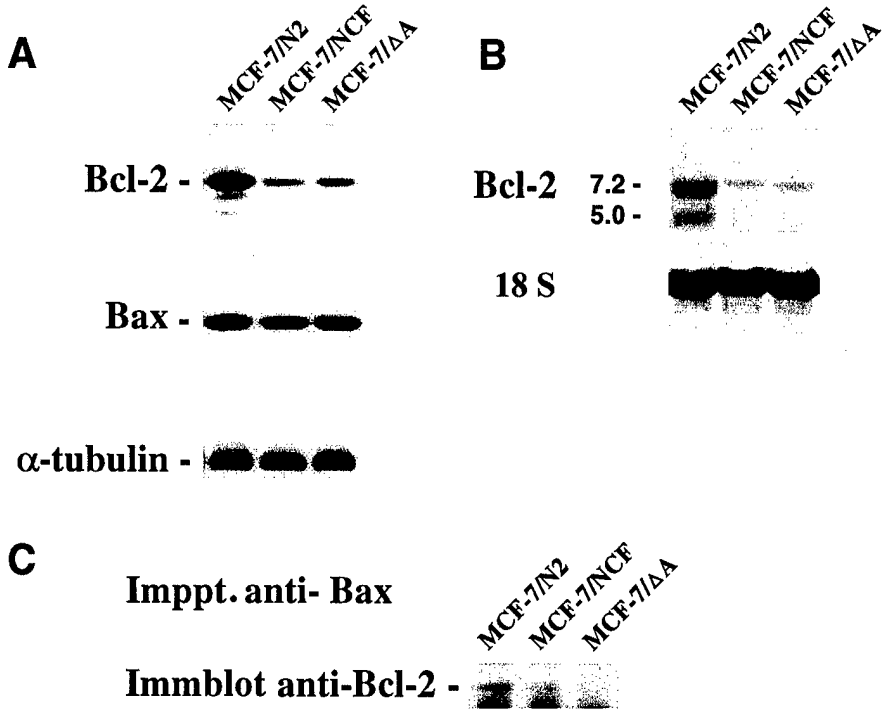


Figure 14. Effect of bFGF expression on Bcl-2 and Bax protein (A) and Bcl-2 mRNA levels (B) in MCF-7 cells. Western blots were reprobed with an antibody to α-tubulin and Northern blots were rehybridized with a ribosomal 18S cDNA probe as loading controls. C. Effects of bFGF expression on co-immunoprecipitation of Bcl-2 with Bax in immunoprecipitation/Western blots. Cells were immunoprecipitated with antibody to Bax and immunostained with antibody to Bcl-2 demonstrating decreased association of Bcl-2 with Bax in cells overexpressing bFGF.

Conclusions

The data presented in this report demonstrate that overexpression of bFGF in MCF-7 human breast cancer cells causes a decrease in Bcl-2 mRNA and protein levels and promotes the susceptibility of MCF-7 cells to undergo programmed cells death in response to chemical injury. These findings are directly opposite those observed in NIH 3T3 cells engineered to overexpress bFGF in which Bcl-2 levels are downregulated and which are protected from the apoptotic effects of serum deprivation and chemotherapeutic drugs. These data suggest different roles for bFGF in the survival of breast cancer cells from those of supporting cells. Whereas bFGF is lost from breast cancer cells as they progress toward more malignant phenotypes, our data suggest that the loss of this intracellular bFGF may play a role in the development of a relative chemoresistance in breast cancer cells. Further studies on the differences in the regulation of Bcl-2 in different cell types will yield valuable information on the control of cell survival by growth factors.

REFERENCES

1. Fenig, E., Wieder, R., Paglin, S., Wang, H., Persaud, R., Haimovitz-Friedman, A., Fuks, Z. and Yahalom, J. (1997) Basic fibroblast growth factor confers growth inhibition and MAP kinase activation in human breast cancer cells. Clinical Cancer Research **3**, 135-142.

2. Wang H, Rubin M, Fenig E, DeBlasio T, Mendelsohn J, Yahalom J and Wieder R. (1997) Basic FGF causes growth arrest in MCF-7 human breast cancer cells while inducing both mitogenic and inhibitory G_1 events. Cancer Research 57: 1750-1757.

3. Armentano, D., Yu, S.-F., Kantoff, P. W., von Ruden, T., Anderson, W. F. and Gilboa, E. (1987) Effect of internal viral sequences on the utility of retroviral vectors. J. Virol. **61**, 1647-1650.

4. Prats, H., Kaghad, M., Prats, A. C., Klagsbrun, M., Lelias, J. M., Liauzun, P., Chalon, P., Tauber, J. P., Amalric, F., Smith, J. A. and Caput, D. (1989) High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. Proc. Natl. Acad. Sci USA **86**, 1836-1840.

5. Florkiewicz, R. Z. and Sommer, A. (1989) Human basic fibroblast growth factor gene encodes four polypeptides: Three initiate translation from non-AUG codons. Proc. Natl. Acad. Sci. **86**, 3978-3981.

6. Preston, G. A., Lang, J. E., Maronpot, R. R. and Barrett, J. C. (1994). Regulation of apoptosis by low serum in cells of different stages of neoplastic progression: Enhanced susceptibility after loss of a senescence gene and decreased susceptibility after loss of a tumor suppressor gene. Cancer Research **54**, 4214-4223.

7. Wang Q, Maloof P, Wang H, Fenig E, Stein D, Nichols G, Denny TN, Yahalom J and Wieder R. Basic fibroblast growth factor (bFGF) downregulates Bcl-2 and promotes apoptosis in MCF-7 human breast cancer cells. (submitted)

8. Ajchenbaum F, Ando K, DeCaprio JA, Griffin JD. Independent regulation of human D-type cyclin gene expression during G_1 phase in primary human T lymphocytes. (1993) J. Biol. Chem. 268:4113-4119.